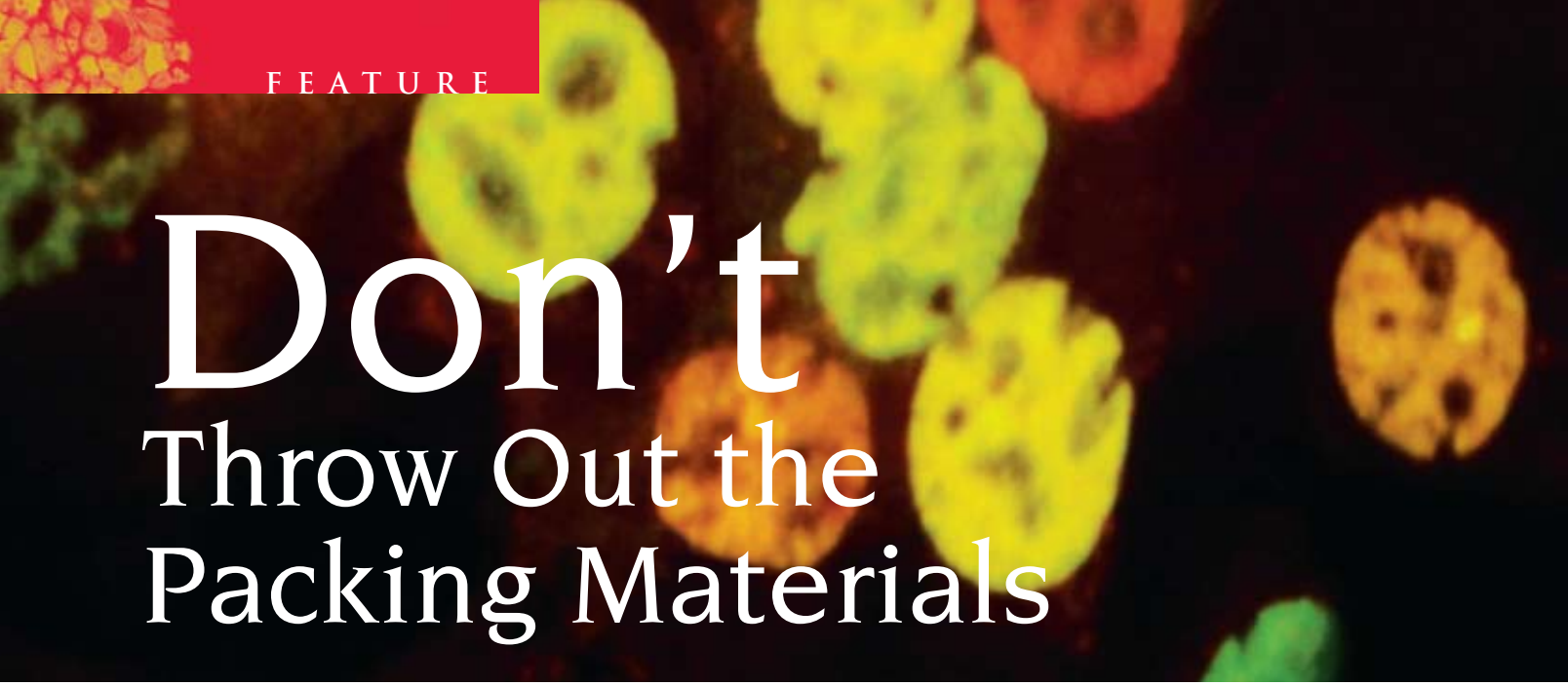


Don't Throw Out the Packing Materials

Most illustrations of DNA depict a kind of ladder spiraling off into the distance, the ladder being the famous DNA double helix consisting of paired nucleotide bases. Although it has long been known that mammalian DNA is packed very tightly and systematically with specialized proteins into material called chromatin, researchers are only now beginning to appreciate the importance of chromatin structure in gene regulation. Gordon Hager, Ph.D., Chief of the Laboratory of Receptor Biology and Gene Expression, has built his considerable scientific achievements on the study of nuclear hormone receptors, using the glucocorticoid receptor (GR) as a prototype. Not without controversy, his research has brought him inexorably closer to the pivotal role and complex dynamics of chromatin structure in the control of gene regulation.

"In the 1980s," recalled Hager, "chromatin was a bit of a dirty word." A wave of experiments done by several different laboratories that were designed to transcribe genes from chromatin fractions had ended in the purgatory of experimental artifacts a few years earlier. Wary scientists shied away from studying chromatin as anything more than DNA packing material. "And there were some groups, including mine, Carl Wu's, Gary Felsenfeld's, and Bob Simpson's that did a lot of the early chromatin work because it simply couldn't get funded extramurally," explained Hager, referring to his current and former NIH Intramural Research Program colleagues.

Hager's laboratory was focused on nuclear hormone receptors—receptors that bind hormones like glucocorticoids, which allow them to interact with particular response elements in the DNA to regulate gene transcription. For reasons that are still only partially understood, the murine mammary tumor virus (MMTV) contains a regulatory element that binds GR when MMTV is integrated into cellular DNA. The team discovered that when MMTV integrated into the mammalian genome, chromatin structural elements called nucleosomes were invariably positioned over the GR binding sites.

This discovery was soon followed by studies showing that GR binds directly to a nucleosome and that, as a result, the nucleosome undergoes a structural transition. To measure the change in chromatin structure, the team used an assay known as DNAse hypersensitivity. DNAse or deoxyribonuclease is an enzyme that will chew up DNA entirely if incubated long enough. However, when incubated only very briefly, DNA is fragmented at easily accessible sites in the chromatin structure that are termed hypersensitive. GR binding sites corresponded to sites of DNA hypersensitivity.

"We proposed that the glucocorticoid receptor was binding to DNA and causing chromatin reorganization at a specifically positioned nucleosome," said Hager.

(*Photo*: R. Baer)

Gordon Hager, Ph.D.

> The structure of chromatin was playing more than just a passive role in gene regulation.

A Few Years in the Doghouse

"In 1987, I proposed at a Keystone Meeting in Park City, Utah, that somehow this chromatin reorganization was part of the mechanism by which the glucocorticoid receptor regulated gene expression," said Hager. Hager was presented with a "Renegade Award" at the meeting, which was not meant as an accolade. "We were in the doghouse," said Hager.

Within a few years, however, the field had shifted its perspective. Evidence began to accumulate suggesting that the structure of chromatin was playing more than just a passive role in gene regulation. A Postdoctoral Fellow in Hager's lab, Trevor Archer, Ph.D., now Chief of the Laboratory of Molecular Carcinogenesis at the National Institute of Environmental Health Sciences, published an influential experiment in 1992 that presented the first direct evidence that the structure of chromatin could prevent a transcription factor from binding to its promoter element. A few years later, C. David Allis, Ph.D., now Tri-Institutional Professor at The Rockefeller University, and colleagues identified a known transcriptional regulator in yeast as an enzyme that modified histone proteins in chromatin.

"And bingo, chromatin was not such a dirty word anymore," said Hager.

It is now clear that remodeling of chromatin is a key event in transcriptional regulation. Many chromatin remodeling factors have been identified and an entire field of epigenetics has emerged to investigate the influence of chromatin modifications on functional gene expression.

There and Back Again

"Most of molecular biology until about the mid-1990s was dead-cell biochemistry," said Hager. "No matter what you do—a DNA footprint or a chip experiment—the first thing you do is kill the cell and then, often, you do 'terrible' things like crosslink the proteins everywhere."

Hager and his colleagues wanted to study gene regulation in living cells. With the advent of green fluorescent protein (GFP), which could be introduced genetically to label proteins of interest, a Postdoctoral Fellow in the lab created GRs tagged with GFP and showed that their fluorescent signature could be visualized under a microscope in living cells.

"And then we remembered cell line 3134," said Hager.

Back in the laboratory's early days, Hager and his team had used the MMTV promoter element as an experimental tool to study glucocorticoid receptor function. "Accidentally, we had a cell line where this structure had amplified itself into a 200-copy tandem array sitting in one place on chromosome 4. That's two million base pairs of DNA with about 1000 GR binding sites in this one place in the chromosome." A more perfectly optimized system for visualizing GR binding could not be readily imagined.

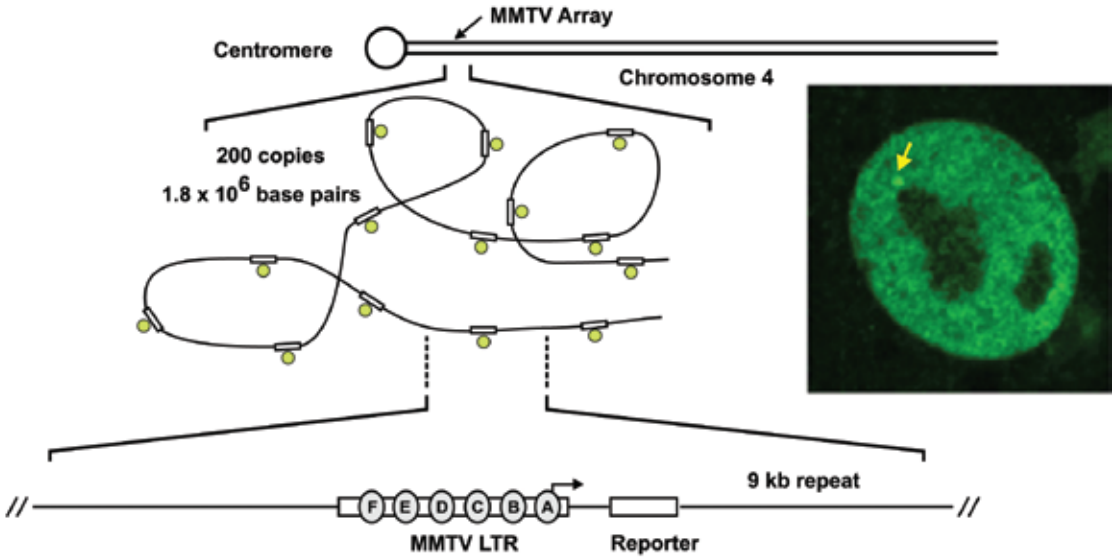
Under a fluorescence microscope, Hager and his colleagues were able to see GRs accumulating on this massive stretch of binding elements. They could also study its kinetics through a technique known as photobleaching. When fluorescent molecules are subjected to light of a particular wavelength, they lose their activity and are no longer visible. By shining a laser on the chromosomal segment, Hager's team could discover whether and when the GRs were replaced by new unbleached molecules.

"We found that they were almost instantaneously replaced," said Hager. "And we were back in the doghouse."

Not only was the result surprising, the idea that DNA binding proteins were operating on such a fleeting timescale contradicted many accepted

> The idea that DNA binding proteins were operating on such a fleeting timescale contradicted many accepted experimental paradigms.

(Image: G. Hager, CCR)



To study nuclear hormone receptor binding, Hager and his colleagues created a cancer cell line with approximately 1000 hormone response elements from the murine mammary tumor virus (MMTV) present in an array on chromosome 4. Activated steroid hormone receptors tagged with green fluorescent protein (GFP) translocate into the nucleus and bind to the array, visualized here as a sharp increase in intensity (yellow arrow) in the cell nucleus.

experimental paradigms for which protein-DNA binding was essentially considered fixed. However, other groups began to do the same kind of experiments with different proteins and the dynamic nature of these interactions gradually came to be accepted.

Hit and Run

To understand the relationship between GR binding and chromatin remodeling, Hager and his colleagues began to look at chromatin remodeling proteins, massive ATP-dependent enzyme complexes that literally grab hold of nucleosomes and alter their higher order structure. Biochemical experiments indicated that the chromatin remodeling protein complex, hSWI/SNF, could create chromatin transitions in the presence of GR. But, they also suggested, paradoxically, that activation of the hSWI/SNF complex disrupted GR binding.

Hager's team decided to use ultrafast UV laser-crosslinking to examine this phenomenon in more detail. They incubated GR and hSWI/SNF with chromatin containing the MMTV array of promoter elements and then studied the resulting interactions at different points in time by taking samples and rapidly crosslinking everything with the UV laser. Their results revealed strong evidence that initially, GR binds to the promoter and recruits the hSWI/SNF complex, but then is displaced during chromatin remodeling.

"The GR proteins are binding to a structure that is being destroyed by the very hSWI/SNF enzyme that it is recruiting," said Hager. "These hSWI/SNF enzymes are giant complexes running around the DNA, so it makes sense that sooner or later they're going to run into the DNA binding protein."

This model of transient, reversible interactions of transcriptional regulators with DNA is now well known as the "hit-and-run" model. It appears to be a central mechanism for all of transcription biology.

Recently, the Hager lab has turned to high-throughput imaging to study the mechanisms by which nuclear receptors migrate to their targets. Using small interfering RNAs (siRNAs) to knock down individual proteins, the team can search for molecules that will prevent migration or clustering of GRs along an MMTV array structure.

Ty Voss, Ph.D., who runs the NCI High-Throughput Imaging Facility, is collaborating with Hager's group and other investigators to optimize and run their assays using high-throughput microscopy. "These microscopes are capable of taking maybe 25-50,000 pictures a day at 300 nanometer resolution, giving very fine details of subcellular organization," said Voss. For siRNA screens, cells are placed on plastic plates with 384 wells, with different siRNAs introduced into each well. An automated liquid handler can process several thousands of such samples in an hour. Voss programs the microscope to automatically analyze features of interest, like the fluorescence generated when GRs bind the MMTV array.

"That's the latest stage in the evolution of this living cell technology. You don't have to know anything about pathways—you could, in principle, look at every gene in the genome," said Hager.

Scaling Up

"About four years ago," explained Sam John, Ph.D., a Staff Scientist in the

> "We decided to stick our toes into the genomics waters and see how cold they were."

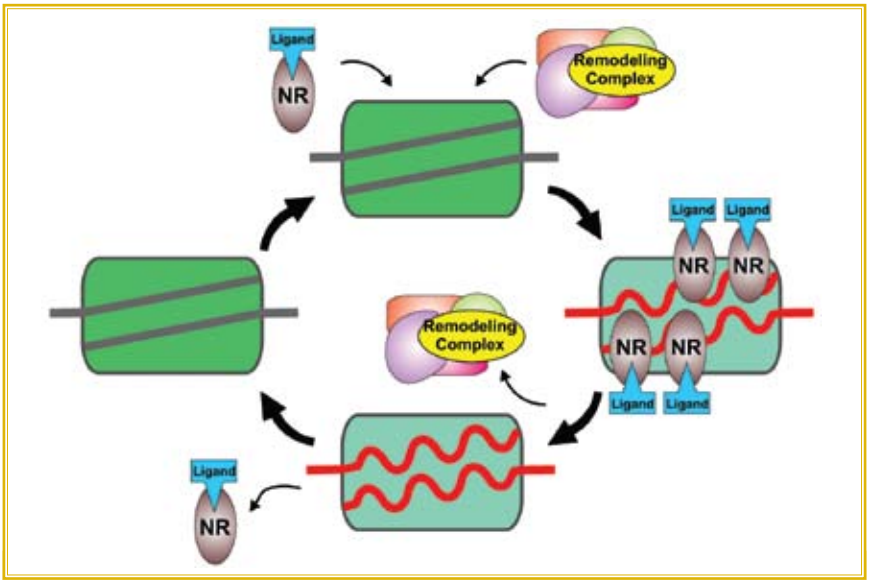
Hager lab, "we decided to stick our toes into the genomics waters and see how cold they were." It turned out that the temperature was just right, and the lab shifted from focusing on one or two genes to studying transcriptional regulation and chromatin structure in the context of the entire genome.

DNase hypersensitivity assays—the technique that Hager and his colleagues used when first suggesting a role for chromatin structure in GR binding and activation—got a boost into the 21st century with the creation of Digital DNase or DHS-Seq by John Stamatoyannopoulos, Ph.D., Hager's collaborator and friend at the University of Washington. Instead of studying a single hypersensitive site, DHS-Seq allows the genome-wide mapping of all the hypersensitive sites in a given cell.

"We found that, with a handful of exceptions, every place the GR protein hits the genome, corresponds with a hypersensitivity site," said Hager. The number of binding sites they were surveying was on the order of 100,000. The surprise came, however, in the order of events. For 85 percent of GR binding sites, the hypersensitivity, i.e. remodeling and opening of chromatin structure, was already present before GR binding.

Furthermore, Hager and his colleagues found that both DNase hypersensitivity and GR binding across the entire genome were dependent on cell type. "When we compared accessible chromatin regions, we found that the organizational overlap was very, very small. And when we looked at where GR bound in cell lines, very different patterns emerged."

"The convention has always been that GR interactions with chromatin result in hormone-dependent changes in chromatin structure," said John. "It turns out that the organization of chromatin at baseline is also an important determinant of how a transcriptional regulator finds and binds its target sites in chromatin." By extension, the structure of chromatin at baseline appears to be important for defining a cell.



(Image: G. Hager, CCR)

Hager and his colleagues have proposed that the recruitment of remodeling complexes by nuclear hormone receptors leads to a transient opening of nucleosome positioning in chromatin. This modified chromatin structure will be accessible to multiple transcription factors during the lifetime of the modified state. After completion of the remodeling cycle, the nucleosome returns to its previous configuation, and a new cycle is initiated.

DNase hypersensitivity sites—not genes—appear to account for 60 to 70 percent of all targets identified in genome-wide disease association studies. That is, many single nucleotide polymorphisms are not located in genes but rather are found in hypersensitivity spots. "This is going to be key in cancer biology—mutations in people that cause dysregulation of their regulatory elements," concluded Hager.

Far, Far Away

Chromatin structure is not just important within localized regions of DNA. Recently, chromosome conformation-capture technologies are being used to identify distant sequences of DNA that come together when DNA forms loops.

"The best data we have is in T cells; as they mature and differentiate, the whole nuclear material gets reorganized," said Hager. Genes that are activated come together in clusters, called hubs. "This is the next frontier in cell biology—how to understand the structure of the nucleus. That takes us back to this dynamic question: If the proteins that are binding to these sites are coming and going so fast, how can they possibly get hold of the DNA for long enough to form a long-range interaction?"

"For our group, the dynamics of chromatin remodeling is a key issue. And it all started back in 1987 with that first experiment that put us in the doghouse," said Hager. "I always wind up at the end of my seminars saying something like 'biology is chemistry.' These are chemical reactions, but we often view them as static macromolecular cartoons… The next breakthrough will come from observations of single molecules moving in living cells."

To learn more about Dr. Hager's research, please visit his CCR Web site at http://ccr.cancer.gov/staff/staff.asp?Name=hager.